compensated decreased blood volume. Epinephrine may antagonize all of those effects by its arteriolar vasoconstrictive action; and in addition protects adrenalectomized animals against histamine and anaphylactic shock. Antihistaminics inhibit egg white edema, hyaluronidase edema,[38] urticarias, angioneurotic edema and histamine and anaphylactic shock probably both by antagonizing the action of released histamine and by decreasing capillary filtration by arteriolar vasoconstriction. No explanation can be given for the protective effect of adrenergic blockade in egg white edema. At first thought, it might have been anticipated that it would aggravate the condition by blocking the protective action of endogenous epinephrine and sympathin-N. This is not the case, however, and the fact the adrenal demedullation also is not detrimental is confirmatory of this lack of effect. It is possible that SFK-501 and the adrenolytic component of SY-28 protect in the same way that Dibenamine does in hemorrhagic and traumatic shock, as demonstrated by Remington,[39,40] although the mode of action in both cases is unknown. The effect suggests, however, the participation of an adrenergic component in egg white edema, as seems to be the case in the pulmonary edemas induced by ammonium chloride[41,42] and increased intracranial pressure or brain concussion.[43]

Summary. Epinephrine protects adrenal insufficient rats from the edema and mortality caused by the intraperitoneal administration of egg white. Mortality is prevented by doses (0.04 mg/kg) which are not able to prevent edema in rats with intact adrenals.

The antihistaminic Phenergan, N-dimethylamino-2-propyl-l-thiodiphenylamine ("3277 RP"), in doses as low as 5 mg/kg prevent death from egg white edema in adrenalectomized rats.

"SY-28", N-(2-bromoethyl)-1-naphthalenemethylamine hydrobromide, a Dibenamine congener possessing both antihistaminic and adrenergic blocking activity, also prevents the edema and death, but is in itself toxic to adrenalectomized rats.

"SKF-501", N-(9-fluorenyl)-N-ethyl-β-chloroethylamine, a Dibenamine cogener with potent adrenergic blocking powers but with a low toxicity and without antihistamine action, also inhibits the edema and decreases the mortality.

The possible modes of action are discussed.

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Received October 18, 1949. P.S.E.B.M., 1949, **72**.

A Microbiological Assay for Vitamin B_{12} Using *Lactobacillus leichmannii*.* (17485)

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Lactobacillus leichmannii (ATCC 4797) has been reported by several laboratories[1-3] to be a useful test organism for the microbiological measurement of vitamin B_{12}. Skeggs, *et al.*[1] found ascorbic acid and air to stimulate a growth response in this organism in the absence of vitamin B_{12} on a basal medium containing casein hydrolysates, but were able to minimize these effects by autoclaving the test assay for 15 minutes at 120°C. Other workers[3] also found certain reducing agents to promote a growth response in both *L. leichmannii* (ATCC 4797) and *L. leichmannii* (ATCC 7830) in the absence

of vitamin B_{12} on casein hydrolysate media, but in addition found that these agents were able to increase the growth response produced by vitamin B_{12}. In attempting to overcome these difficulties, a microbiological assay has been developed which is highly sensitive to vitamin B_{12} and which yields consistent results. The experimental basis and the details of the procedure are presented in this report.

Assay Procedure. The culture of *Lactobacillus leichmannii* 4797 used in this assay was obtained from the American Type Culture Collection, Georgetown University, Washington, D. C. *L. leichmannii* (ATCC 7830) has also been used successfully in this assay. The culture is maintained in a stab medium composed of 5 ml of double strength basal medium (Table I), 0.3 unit of 15 unit USP injectable liver extract, 150 mg of agar and distilled water to make 10 ml. Each week it is transferred to a broth medium[4] in which it is grown for 24 hours at 37°C and is then taken back to the stab medium. The inoculum is prepared by growing the organism for 24 hours in a broth medium having the same composition as the stab medium except for the omission of agar. The procedure for the inoculation and incubation of the assay is the same as that described by Daniel, *et al.*[5] except that the diluted inoculum is made to read 60 on the galvanometer scale of a Coleman spectrophotometer at wave length 650 mμ. The composition of the amino acid medium used in this method is presented in Table I. It is a modification of the medium used by Daniel, *et al.*[5] in studies on the nutrition of *L. casei* and that of Snell, *et al.*[6] for *L. leichmannii.*

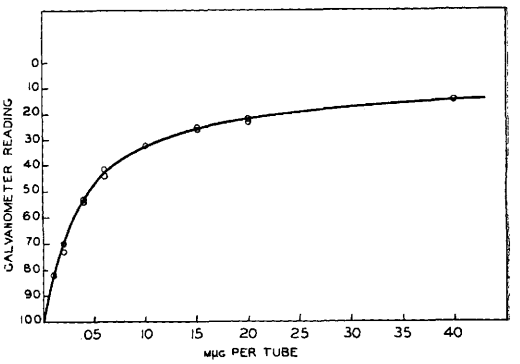

FIG. 1.

Response of *Lactobacillus leichmannii* to crystalline vit. B_{12}.

After all constituents of the medium are combined, the pH is adjusted to 5.5 with 20% potassium hydroxide. The volume is then made to 1 liter and the medium steamed for 5 minutes. Five ml of the double-strength basal medium and the sample to be assayed are placed in the assay tubes. The final volume is made to 10 ml. The assay is incubated at 37°C for 16 hours or until the tube containing 0.1 mγ of vitamin B_{12} reaches a turbidimetric reading of approximately 30 on the galvanometer scale of the spectrophotometer. At this time the turbidity of the cell suspension is read by placing the inoculated blanks at 100 on the galvanometer scale. Very little growth, if any, is ordinarily present in the blanks. A typical standard curve is shown in Fig. 1. The results show that on this basal medium the assay range is between 0.001 and 0.04 mγ of vitamin B_{12} per ml of medium.

The canned tomato juice used in this method is purchased on the open market. It is filtered through No. 617 Eaton and Dikeman†

* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D.C., and was aided by grants to Cornell University by the Nutrition Foundation, Inc., New York, the Cerophyl Laboratories, Inc., Kansas City, Mo., The Commercial Solvents Corporation, New York City, The Cooperative G.L.F. Exchange, Ithaca, N. Y., and the Western Condensing Company, San Francisco, Calif. This work was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry. The technical assistance of Betty F. Brown and Diana M. Cameron is acknowledged.

1 Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem.*, 1948, **176**, 1459.

2 Capps, B. F., Hobbs, N. L., and Fox, S. H., *J. Biol. Chem.*, 1949, **178**, 517.

3 Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffman, C. E., Hutchings, B. L., and Jukes, T. H., *Fed. Proc.*, 1949, **8**, 257.

4 Nymon, M. C., Gunsalus, I. C., and Gortner, W. A., *Science*, 1945, **102**, 125.

5 Daniel, L. J., Scott, M. L., Heuser, G. F., and Norris, L. C., *J. Biol. Chem.*, 1948, **174**, 71.

6 Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, 1948, **175**, 473.

filter paper on a Büchner funnel with the aid of Celite filter aid (Johns-Manville). The resulting solution is adsorbed with 12.5 g of activated norit per liter at the natural pH of the serum (4.0 to 4.2) for ½ hour and filtered to remove the norit.

Development of Assay. Crystalline vitamin B_{12}‡ and a potent vitamin B_{12} concentrate[7] were used in the development of this assay. The vitamin B_{12} concentrate was prepared from 15 unit USP injectable liver extract by the technic of counter-current distribution as described by Craig.[8] The results of a study showing the need of *L. leichmannii* for a growth factor present in tomato juice is presented in Table II. Crystalline vitamin B_{12} was observed to produce a submaximal plateau response when assayed on the amino acid medium containing no tomato juice. The addition of adsorbed tomato juice filtrate promoted a much improved growth response from the vitamin especially at the higher levels. Increasing the content of ferrous sulfate and cysteine, or adding ascorbic acid or sodium thioglycollate to the medium, did not replace the need for the supplement contrary to the findings of Stokstad, *et al.*[3] The same results were obtained when lyophilized tomato juice filtrate was employed.

Preliminary experiments showed also that a phenol-n-butyl alcohol extract of USP injectable liver extract which had been freed of vitamin B_{12} and thymidine is capable of replacing tomato juice as a source of unknown factors in the assay medium. Other studies indicated that the factor for *L. leichmannii* and that reported by Daniel, *et al.*[5,9] for *L. casei* are concentrated by counter-current distribution in the same fractions of injectable liver extracts and therefore may be identical. Whether this factor is the same as that reported by Shorb[10] has not been determined.

During some early studies it was found that better growth is obtained with *L. leichmannii* at the same level of vitamin B_{12} when the initial pH of the medium was more acidic than the usual value of 6.6 to 6.8.[1,2,6] In

TABLE I.
Double-Strength Medium* for Assay of Vit. B_{12} Using *Lactobacillus leichmannii.*

DL-Alanine	425	mg
L-Arginine	425	"
DL-Aspartic acid	425	"
L-Cystine	400	"
L-Glutamic acid	1213	"
Glycine	425	"
L-Histidine	425	"
L-Hydroxyproline	50	"
DL-Isoleucine	425	"
DL-Leucine	425	"
L-Lysine	213	"
DL-Methionine	425	"
DL-Norleucine	425	"
DL-Phenylalanine	100	"
L-Proline	425	"
DL-Serine	50	"
DL-Threonine	50	"
DL-Tryptophan	50	"
L-Tyrosine	400	"
DL-Valine	425	"
Asparagine	150	"
Glutamine	200	"
Glucose	40	g
Cysteine hydrochloride	2	"
Sodium acetate (trihydrate)	12	"
Sodium citrate	10	"
$MgSO_4 \cdot 7H_2O$	2.8	"
$FeSO_4 \cdot 7H_2O$	800	mg
$MnSO_4 \cdot 4H_2O$	600	"
KH_2PO_4	4	g
K_2HPO_4	4	"
Adenine sulfate	10	mg
Guanine hydrochloride	10	"
Uracil	10	"
Xanthine	15	"
Tween 80†	2	ml
Tomato juice filtrate‡	600	"
Pyridoxine	4	mg
Pyridoxal	4	"
Niacin	2	"
Riboflavin	2	"
Thiamine	2	"
Calcium pantothenate	2	"
Biotin	10	γ
Folic acid	110	"
Pyridoxamine	800	"
p-Aminobenzoic acid	80	"
Distilled water to make	1000	ml

* After preparation the pH of the medium is adjusted to 5.5 with 20% potassium hydroxide, steamed 5 minutes and cooled.

† Polyoxyethylene sorbitan monooleate (Atlas Powder Co.).

‡ Prepared as described previously.

† The Eaton-Dikeman Company, Mt. Holly Springs, Pa.

‡ The crystalline vitamin B_{12} used in this work was kindly supplied by Dr. E. Lester Smith of Glaxo Laboratories, England, and Dr. D. F. Green of Merck and Co., Rahway, N. J.

7 Carlson, C. W., Thesis, Cornell University.

8 Craig, L. C., *J. Biol. Chem.*, 1944, **155**, 519.

9 Daniel, L. J., Peeler, H. T., Norris, L. C., and Scott, M. L., *J. Biol. Chem.*, 1949, **177**, 917.

10 Shorb, Mary S., *J. Bact.*, 1947, **53**, 669.

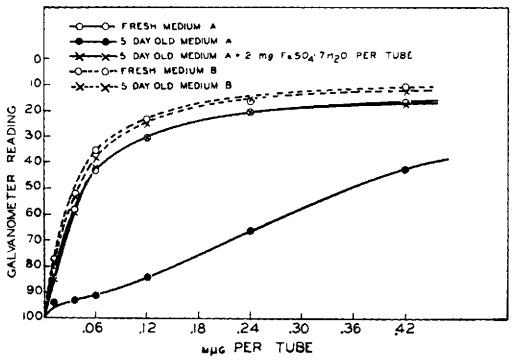

FIG. 2.

Response of *Lactobacillus leichmannii* to vit. B_{12} concentrate on fresh and aged media. Medium A contains no cysteine and only 1.11 mg per tube of $FeSO_4 \cdot 7H_2O$. Medium B contains 4 mg per tube of $FeSO_4 \cdot 7H_2O$ and 10 mg per tube of cysteine.

view of this finding, studies were undertaken to establish the optimum pH range for this organism on the amino acid medium. Nine samples of the medium were used. Each contained vitamin B_{12} in such quantity that, on dispensing, every assay tube had 0.4 mγ of vitamin B_{12} . The pH of these samples was adjusted so as to have a graded series ranging from 4.0 to 7.0. The results showed that under these conditions maximum growth was progressively retarded at pH values greater, or less, than pH 5.5.

On a medium prepared as shown in Table I, but containing no cysteine and only 222 mg of $FeSO_4 \cdot 7H_2O$ per liter, poorer growth was obtained at the same level of vitamin B_{12} when the medium was a few days old than when it was prepared fresh. This effect became marked if a vitamin B_{12} concentrate prepared from a 15 unit USP injectable liver extract was used as the supplement. In attempting to overcome the effect of aging, the addition of 2 mg of ferrous sulphate per tube to the aged medium was found to restore it to a "fresh" condition. Response curves demonstrating this are shown in Fig. 2.

Studies were then undertaken to determine how to preserve the medium in a fresh condition for longer periods of time. In these experiments 0.118 mγ of vitamin B_{12} concentrate per tube was assayed in the presence of graded levels of $FeSO_4 \cdot 7H_2O$ and cysteine. The results of the study showed that the growth response reached a plateau with either 4 mg of ferrous sulfate, or 15 mg of cysteine per tube. A combination of 4 mg of ferrous sulfate per tube and graded levels of cysteine, however, promoted an additional growth response which reached a plateau with only 4 mg of cysteine per tube. Additional cysteine had no effect on growth but aided in maintaining the medium in a fresh state for a longer period of time. As a consequence of these findings, the composition of the medium was changed so that it contains 4 mg of $FeSO_4 \cdot 7H_2O$ and 10 mg of cysteine per tube. The effect of allowing the medium containing the increased iron and cysteine to age 5 days before use is also shown in Fig. 2.

Certain discrepancies were found to exist between the assays conducted on the amino acid medium described in this report and those conducted on a medium prepared as described by Skeggs, *et al.*[1] The responses of crystalline vitamin B_{12} and a vitamin B_{12} concentrate using these media are presented in Table III. The values from the amino acid medium were obtained at 16 hours and those on the casein hydrolysate medium at 24 hours.[1] In each case more growth resulted from the same level of vitamin B_{12} on the amino acid medium than on the other. Furthermore, the vitamin B_{12} concentrate which was shown to be highly active for the chick[11] promoted only slight response on the casein hydrolysate basal medium. On investigating the cause of this discrepancy it was found that addition of ferrous sulfate to the casein hydrolysate medium markedly increased the growth response from the vitamin B_{12} concentrate which is in agreement with the work of Stokstad, *et al.*[3] The assay blanks were found to have considerable growth under these conditions. This growth was found to be due to the hydrolyzed casein in the medium and indicated that casein hydrolysate contained a form of vitamin B_{12} active only in the presence of reducing agents. This is probably the same effect noted by Welch, *et al.*[12]

11 Carlson, C. W., Miller, R. F., Peeler, H. T., Norris, L. C., and Heuser, G. F., *Poultry Sci.*, 1949, **28**, 750.

12 Welch, A. D., and Wilson, F. F., *Arch. Biochem.*, 1949, **22**, 486.

TABLE II.
Response of *L. leichmannii* to Vit. B_{12} in Presence and Absence of an Unidentified Growth Factor in the Medium.

Substance	Level per tube mγ	Galvanometer readings*	
		Medium without tomato juice filtrate	Medium with tomato juice filtrate
None		100	100
Crystalline vit. B_{12}	.012	85	80
	.036	75	57
	.059	66	42
	.118	57	26
	.236	50	20
	.412	53	13

* A galvanometer reading of 100 represents no growth.

TABLE III.
Comparison of Two Different Media in the Assay of Vit. B_{12}.

Substance	Level per tube, mγ	Galvanometer readings*	
		Amino acid medium†	Casein-hydrolysate medium‡
None		100	100
Crystalline vit. B_{12}	.012	76	95
	.036	49	88
	.059	35	79
	.118	26	61
	.236	16	40
	.412	13	24
Vit. B_{12} conc.§	.012	74	98
	.036	50	93
	.059	36	90
	.118	23	93
	.236	15	83
	.412	11	74
	mg		
Adsorbed enzyme digested casein‖	25	18	—

* A galvanometer reading of 100 means no growth.
† The amino acid medium reported in this paper.
‡ Prepared as described by Skeggs *et al.*, *J. Biol. Chem.*, 1948, **176**, 1459.
§ Prepared as described previously.
‖ A constituent of the casein-hydrolysate medium. Adsorbed three times the amount reported necessary by Skeggs *et al.*, *J. Biol. Chem.*, 1948, **176**, 1459.

The results of studies on the recovery of vitamin B_{12} from USP injectable liver extract are presented in Table IV. The results showed a mean recovery of 100.8% from this product. These results were confirmed by conducting recoveries from Wilson's liver fraction L. In this case individual recoveries ranged from 96.7 to 109.4% with a mean recovery of 100.7%. No synergism was found to exist between the natural materials and crystalline vitamin B_{12}. The assay method, therefore, appears to be specific for vitamin B_{12} activity. Further evidence of this was obtained in counter-current distribution studies of injectable liver extract since the assay of the fractions in the various tubes indicated the partition of a single substance.

Thymidine,§ which was shown to promote

§ The thymidine used in these studies was kindly supplied by Dr. William Shive of the University of Texas.

TABLE IV.
Recoveries of Crystalline Vit. B_{12} Added to Liver Fractions.

Substance	Level per tube, units	Vit. B_{12} by assay, mγ	Added B_{12} per tube, mγ	Theoretical B_{12} per tube, mγ	Actual B_{12} per tube, mγ	Recovery, %	Standard error of the mean
USP injectable liver extr. (15 unit)	.000012	.008	.012	.020	.021	105.0	
	.000025	.012	.012	.024	.026	108.3	
	.00005	.025	.012	.037	.035	94.6	
	.000075	.037	.012	.049	.051	104.1	
	.0001	.051	.012	.063	.058	92.1	
						100.8	± 3.154

TABLE V.
Uniformity of Vit. B_{12} Assay Values Obtained from Various Substances with *L. leichmannii*.

Substance	No. of assays	Vit. B_{12} content	Range	Standard error of the mean	Coefficient of variation, %
Vit. B_{12} conc.*	14	8.06 γ/ml	7.69 –8.54	± 0.067	3.10
USP injectable liver extr. (15 unit)	10	0.590 γ/unit	0.545–0.629	± 0.009	4.64
Wilson's liver L	7	0.389 γ/g	0.374–0.416	± 0.006	3.62

* Prepared as described previously.

a growth response in *L. leichmannii* on a casein hydrolysate medium,[1] was also found to promote a growth response on the amino acid medium. Twenty-four thousand times more thymidine than vitamin B_{12} was required to produce the same growth response. Due to this wide ratio, it is highly improbable that interference from thymidine will be encountered in the assay of most materials for vitamin B_{12} activity by the procedure described in this report.

The ability of the method to produce repeatable assay results was determined by running several assays on successive days on the same sample of unknown against crystalline vitamin B_{12} solution. The results are presented in Table V. Excellent replicability was found to exist between repeated daily assays. Values from a crude liver fraction were just as consistent as those from a vitamin B_{12} concentrate.

Assay of Various Materials. Feedstuffs currently employed in experimental chick diets used to study vitamin B_{12} at this laboratory were chosen for the development of extraction procedures. The vitamin B_{12} content of several of these materials is presented in Table VI.

USP injectable liver extracts were found to need no treatment other than dilution. A range of 87.1 to 2170 mγ of vitamin B_{12} per USP unit was found from the assay of 10 different products. The results confirm the report by Rickes, *et al.*[13] that a considerable variation exists in the amount of vitamin B_{12} to which 1 USP unit is equivalent. In the liberation of vitamin B_{12} from animal products 1 g sample is placed in a 125 ml Erlenmeyer flask together with 50 ml of phosphate buffer (pH 6.8), 20 mg of pancreatin suspended in phosphate buffer, and 1 ml of toluene. The materials are well mixed and incubated for 20 hours at 37°C. After incubation the pH is adjusted to 5.5 with 2 N HCl followed by 5 minutes of steaming. The sample is cooled, adjusted to 100 ml and filtered through Whatman fluted filter paper No. 12. With vegetable materials a 2 g sample is placed in a 250 ml Erlenmeyer flask together with 100 ml of acetate buffer (pH 4.5). The mixture is autoclaved for 30 minutes at 20 lb. After cooling, a suspension containing 40 mg of papain and 40 mg of

[13] Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **107**, 396.

TABLE VI.
Vit. B_{12} Content of Several Materials.

Material	Vit. B_{12} content, mγ/g
Soybean meal	11
Yellow corn	10
Wheat	7
Alfalfa leaf meal	40
Dried brewers' yeast	8
Wilson's liver L	389
Crude casein	104
White fish meal	98.3
Red fish meal	111
Condensed fish solubles	92
Meat scraps	42.3

takadiastase is added to the mixture together with 2 mg of neutralized cysteine hydrochloride and 1 ml of toluene. The materials are well mixed and incubated for 40 hours at 37°C. After incubation the samples are adjusted to pH 5.5, steamed 5 minutes, cooled, adjusted to 200 ml and filtered through Whatman fluted filter paper No. 12.

Other treatments used in an attempt to liberate the vitamin include autoclaving at acidic and neutral pH, allowing the samples to autolyze for 20 hours in acetate buffer, steaming 30 minutes in acetate buffer and allowing the sample to stand in distilled water. These treatments did not liberate as much vitamin B_{12} as was obtained by the methods outlined in detail. Treatment of the animal products with trypsin or pepsin produced the same results as were obtained with pancreatin. However, pancreatin is used in the procedure because less growth occurred in the enzyme assay blanks.

Summary. A microbiological assay for vitamin B_{12} using *Lactobacillus leichmannii* (ATCC 4797) has been developed. The assay medium contains crystalline amino acids as the nitrogen source and adsorbed tomato juice filtrate as a source of unidentified growth factors. A high level of cysteine and ferrous sulfate are present in the medium to keep it in the necessary reduced state. Data are given to show the degree of precision and reproducibility to be expected from the assay.

Received Sept. 3, 1949. P.S.E.B.M., 1949, **72**.